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Designing heparan sulfate-based biocompatible polymers and their application for intracellular stimuli-sensitive drug delivery

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ABSTRACT

Heparan sulfate (HS) is a kind of natural polysaccharides with good biocompatibility. And as drug carriers, it has some advantages compared to heparin. However, the preparation of HS is cumbersome and difficult, which limits its application in drug delivery. Here, we use modern separation technique combined with chromatography to establish a new preparation method of HS. The molecular weight and degree of dispersion of HS were $(1.03 \times 10^4 \pm 107)$ kDa and 1.106, respectively. HS also showed low anticoagulation activity in comparison with heparin. Subsequently, novel redox-sensitive heparan sulfate-cystamine-vitamin E succinate (HS-SS-VES, HSV) micelles were designed to increase tumor selectivity and improve the therapeutic effect of doxorubicin (DOX). DOX-loaded HSV micelles (DOX/HSV) with spherical morphology had average particle size of 90–120 nm and good redox-triggered release behavior. The cell viabilities of blank micelles were > 90% in both human breast cancer (MCF7) cells and African green monkey SV40-transformed kidney fibroblast (COS7) cells. However, the cytotoxicity of DOX/HSV in MCF7 cells was higher than that of COS7 cells. Flow cytometry analyses and confocal laser scanning microscopy observation indicated that DOX/HSV micelles were internalized by endocytosis, and then the drug was released quickly and entered the nuclei of tumor cells. The results demonstrate that high-purity HS can be prepared and has the potential to be further used for drug delivery in antitumor applications.

1. Introduction

Treatment and control of cancer increasingly becomes a serious global health issue. Among various therapeutic options, chemotherapy is still one of the first-line treatment strategies for malignant tumors [1]. Doxorubicin (DOX) with high efficiency in several cancer treatments is one of the most important chemical drugs. However, DOX can lead to serious side-effects such as nephrotoxicity and cardiotoxicity, and the critical part for the success of chemotherapy is whether DOX can be effectively delivered into tumor tissue instead of rapid blood clearance and serious side-effects on normal tissues [2,3]. So the developments of DOX delivery systems have been reported [4]. Micelles have been widely researched in anticancer treatment to maximize therapeutic efficacy and reduce side effects [5,6]. The micelles are formed by self-assembly of amphiphilic conjugates, and they can prolong blood circulation time, increase the solubility and target tumors with enhanced permeability and retention (EPR) effect [7]. Because of these advantages, micelles have become one of the most promising drug delivery systems for controlling the release of various anticancer drugs [8–13].

After reaching the tumor cells, the drugs must be released from micelles before they can play therapeutic effects [14]. In the past decade, in order to increase the drug release in tumor cells, different smart carriers with responses to various tumors microenvironments were developed. Stimuli-sensitive nanoparticles can be triggered by pH, temperature, enzyme, redox potential, light, magnetic field and ultrasound [15–21], resulting in rapid release of the loaded drugs. Among these stimuli-sensitive nanocarriers, redox-trigger nanoparticles have been widely studied for drug delivery because of the difference in redox potentials between the tumor cells and extracellular environment [22–24]. Glutathione (GSH), a thiol-tripeptide being able to reduce disulfide bonds, is rare in extracellular environment (approximately 10^{-6}–10^{-5} M) but abundant in the tumor cell cytosolic (approximately 10^{-3}–10^{-2} M) [25]. It is worth-noting that the concentration of GSH in

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many tumor cells is higher than that in normal cells [26–29]. The concentration differences of GSH can trigger the rapid release of active components such as drugs, genes and proteins from the redox-sensitive nanocarriers in tumor cells [30,31]. Therefore, a large amount of redox-sensitive micelles have been investigated for fast drug release and enhanced efficacy [32–35].

The natural polysaccharides in mammals have attracted wide attention as drug carriers because of their many inherent advantages such as low toxicity, good biodegradability and high biocompatibility [36,37]. Furthermore, the special reactive terminal groups make polysaccharides possible for chemo-selective modifications, while maintaining their intrinsic biological properties [38]. Therefore, hydrophobic modifications of natural polysaccharides to prepare micelles have been carried out in recent years. Heparin polysaccharides have been often used to deliver anti-tumor drugs. Lee J S et al. [39] applied quaternary acid and poly-ε caprolactone to decorate heparin to synthesize amphiphilic conjugates, which could form a long-acting injection for delivery of basic fibroblast growth factor. However, heparin with too high anticoagulation effect may cause spontaneous hemorrhage and other adverse reactions, which seriously affected the use of heparin. Moreover, its strong electronegativity may hamper its entering into cells. Compared with heparin, heparan sulfate (HS) with different proportion of disaccharide sequences has low anticoagulant activity. Furthermore, HS cannot be easily attacked by macrophages or complements systems, and therefore has a long circulatory effect. In recent years, studies have also found that HS can inhibit tumor cell proliferation, tumor invasion and metastasis and also can inhibit the formation of neovascularization in tumor tissues [40]. Though HS has many advantages, the traditional preparation of HS is inefficient and cumbersome, resulting in low purity and difficulty in purchase, which highly limited the design of HS-based drug delivery systems.

Herein, we used modern separation technique combined with chromatography to prepare high-purity HS. Then, we developed HS-based reduction-sensitive polymer micelles (HSV) for the delivery of DOX (Scheme 1). The reduction-insensitive micelles (HDV) formed from an analogous structure were also prepared as control. The reduction-sensitive character of the micelles was investigated by *in vitro* triggered drug release and dynamic light scattering (DLS). The cytotoxicity of blank micelles and drug-loaded micelles against MCF7 cells and COS7 cells was evaluated by the MTT assay. The intracellular release in MCF-7 cells and COS7 cells was further demonstrated by flow cytometry (FCM) and confocal laser scanning microscopy (CLSM). And the internalization pathways of DOX-loaded micelles in MCF7 cells were also investigated by FCM.

2. Experimental section

2.1. Materials

Crude heparin (Shandong, China), D-α-tocopherol acid succinate (VES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide were purchased from Aladdin Regent Co. (Shanghai, China). 1,6-Diaminohexane dihydrochloride (DIA), cystamine dihydrochloride (CYS) were provided by Energy Chemical Reagent Co. (Shanghai, China). Dimethyl sulfoxide (DSMO), glutathione (GSH), penicillin–streptomycin solution, and pancreatin were obtained from Shanghai Songan Biotech Co. Ltd. (Shanghai, China). Tetrahydrofuran (THF), formamide, dimethylformamide (DMF), triethylamine (TEA) and tris(hydroxymethyl)methyl aminomethane hydrochloride (Tris·HCl) were obtained from Shanghai Reagent Chemical Co. (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Dalian Meilune Biotech Co. Ltd. (Dalian, China). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and RPMI-1640 medium were purchased from Gibco BRL (Maryland, USA). 3-[4,5-Dimethylthiazol-2-yl]-2, 5-diphenyltetrazoliumbromide (MTT) was obtained from Invitrogen Corp. 4, 6-diamidino-2-phenylindole (DAPI) were obtained from Shanghai Beyotime Biotechnology Co. Ltd. (Shanghai, China). All other reagents were of analytical grade.

2.2. Cell culture

Human breast cancer (MCF7) cells and transformed African green monkey SV40-transformed kidney fibroblast (COS7) cells were purchased from Chinese Academy of Sciences (Shanghai, China) and grown in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin sulfate, 100 U/mL penicillin G sodium. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of heparan sulfate (HS)

Crude heparin mainly contains heparin, but also contains some HS, a small amount of dermatan sulfate and a very low content of chondroitin sulfate, and these polysaccharides have sulfate groups, carboxylic acid groups and negative charges. According to the differences in the molecular weight and number of charges, HS was prepared by an anion exchange chromatography column on AKTA purification system (General Electric, USA). Tris-HCl buffer (20 mmol/L, pH 7.4) was used as the mobile phase and different concentrations of sodium chloride solution were prepared as eluents.

The prepared HS was further purified by the method of ethanol fractional precipitation to improve the purity. Briefly, the sample and sodium chloride were dissolved in deionized water, and ethanol was added stepwise from 0.1 Volume to 1.5 Volume and the bottom sediment was centrifuged. The precipitate was dissolved, dialyzed for three days, and then freeze-dried and stored. Different components were analyzed by agarose gel electrophoresis. The ultrafiltration membrane with a molecular weight cut-off of 10 kDa was selected to further desalinate and remove small molecular impurities and improve the dispersibility of HS.

2.4. Characterization of HS

The molecular weight of HS was determined by multi-angle laser light scattering gel chromatography system (SEC-MALLS, Wyatt, UK). Sodium chloride solution (0.02 mol/L) was used as the mobile phase at a flow rate of 0.5 mL/min. Differential refractive index detector and laser light scattering detector (DAWN HELEOS II) were used. Samples were prepared at a concentration of 5 to 10 mg/mL and injected into the injector. The results were analyzed using ASTRA V6.1.1.17 software.

Heparinases can degrade glycosidic bonds between glucosamine and uronic acid to produce unsaturated disaccharide fragments. Calcium acetate solution (pH 7.0) and the mixed solution of heparin I-II-III were added to the sample solution. Degraded disaccharide fragments were analyzed by strong anion exchange-high performance liquid chromatography (SAX-HPLC). The mobile phase A was 0.028% phosphoric acid-disodium hydrogen phosphate buffer solution (pH 3.0) and mobile phase B was 14% phosphoric acid-perchloric acid buffer solution (pH 3.0). Sulfate and carboxylate molar ratio was calculated indirectly according to the conductance change.

Anticoagulant activity was determined by using chromogenic substrate method. Blank solution, test solution and standard dilution (V, 20 μl–50 μl) were added to different tubes, respectively. Antithrombin solution (V, 20 μl–50 μl), Xa factor solution (2V, 40 μl–100 μl) and chromogenic substrate solution (2V, 40 μl–100 μl) were added to each tube in order. Then, 2% citric acid solution (2V, 40 μl–100 μl) was added to terminate the reaction. The absorbance of each well was determined by microplate reader. There was no significant difference between the two blank wells. The standard curves of anti-factor Xa and anti-factor IIa were respectively prepared, and the anticoagulant